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PRINCIPAL INVESTIGATOR: Stefan M. Pulst, M.D.

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
                           Los Angeles, California 90048

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Stefan M. Pulst, M.D.

E-Mail: pulst@cSHS.org

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14. ABSTRACT

Neurofibromatosis 2 (NF2) is a tumor suppressor gene syndrome characterized by the development of tumors of Schwann cell, meningeal, and ependymal origin. NF2 is also the gene most commonly mutated in sporadic tumors of these cell types. With previous support from the NF2 program of the Army Medical Branch we have identified hepatocyte growth factor regulated kinase substrate (HRS) as a protein that interacts with schwannomin. Studies by us and others have indicated a role for HRS in growth factor receptor trafficking and downregulation of signaling, but also established a role for HRS in cytokine and IGF1-mediated signaling to the STAT pathway. In the first year of funding, we have continued our in vitro studies of Hrs partial proteins and have initiated mouse studies to test interactions. We have identified several different Hrs molecules that show dominant negative effects. Our initial intercrosses of Hrs+/- mice with Nf2+/- mice have been successful. However, we have noticed slightly reduced litter size and some evidence for segregation distortion. This may influence our overall timetable in that it may be more costly and lengthy to produce the number of animals necessary for analysis.

15. SUBJECT TERMS
Neurofibromatosis 2 (NF2), animal models, growth factor receptors

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Introduction:

Although autosomal dominant NF2 is relatively rare with an incidence of 1 in 40,000 (Evans et al., 1992), studies by us and others have demonstrated that virtually all sporadic schwannomas and 50% of ependymomas and meningiomas harbor NF2 mutations (Rubio et al., 1994, Ruttledge et al., 1994; Sainz et al., 1994; Bijlsma et al., 1994; Papi et al., 1995; Lekanne-Deprez et al., 1994, Slavc et al, 1995). NF2 mutations have been identified in tumors and tumor cell lines in a variety of other human tumors. These include breast and lung cancer, melanoma, and mesothelioma (Bianchi et al., 1994, 1995; Sekido et al., 1995). Especially in mesotheliomas, highly malignant asbestos-induced cancers of the pleura, NF2 mutations have repeatedly been found to contain NF2 mutations and loss of NF2 protein (Cheng et al., 1999).

The investigation of NF2 function has been greatly aided by the identification of NF2-interacting proteins and the development of animal models. This proposal has for the first time combined these two approaches. We have identified HRS as an interactor of the NF2 protein. HRS and schwannomin interact physically and functionally.

When we identified HRS as an NF2 interactor, relatively little was known about this protein. It had been identified as a protein phosphorylated in response to treatment of cells with Hepatocyte Growth Factor (HGF, also called scatter factor) (Komada & Kitamura, 1995). It is a 775 amino acid protein with a predicted MW of 110 kDa. Using antibody-labeling and subcellular fractionation it was shown that HRS had an almost ubiquitous expression pattern and was localized to the outside of endosomes. Furthermore, homology to the yeast protein Vps27p, which is essential for protein traffic through a pre-vacuolar compartment in yeast, suggested a role for HRS in vesicular transport through early endosomes (Odorizzi et al., 1998; Komada et al., 1997). Studies by us and others have indicated a role for HRS in ERB receptor trafficking and downregulation of EGFR signaling, but also in cytokine and IGF1-mediated signaling to the STAT pathway. HRS has been shown to be important in signaling in pathways that are relevant to Schwann cell differentiation and proliferation.

Although great strides have recently been made to re-introduce *NF2* into human NF2 schwannoma cells *in vitro*, results between the two groups that pioneered these approaches are only partially overlapping (morphologic changes vs. apoptosis). The difficulties in generating a schwannoma phenotype in *vitro* may be in part related to the fact that Schwann cell proliferation and differentiation are regulated by various signals including axonal electrical activity, adhesion to axons, basal lamina, and adjacent Schwann cells. In addition, phosphorylation status, isoform expression and subcellular localization of schwannomin vary depending on conditions and cellular context *in vitro*; the relevance of these variables is not known *in vivo*.

Animal models for NF2 have been very successful with two major caveats. *Nf2*$^{+/-}$ mice do develop tumors, but do not develop the tumor types typical for human NF2 patients, i.e. schwannoma, meningiomas, or ependymomas. When NF2-deficiency is targeted to Schwann cells, schwannomas develop, but usually relatively late.

By crossing *Nf2*$^{+/-}$ mice with *Hrs*$^{+/-}$ mice we hope to produce a tumor spectrum that includes the development of schwannomas. We will also target HRS-deficiency to Schwann cells using expression of dominant-negative *HRS* alleles. We expect to generate an animal model that will develop schwannomas more reliably and potentially earlier than currently available models.

Body

General Comments:

We experienced significant delays in initiating the funding of this award. Although the award was made in January 2005, internal accounts were only set up in March of 2005. At that time, we also received notification that our animal studies had to undergo an independent review by the USAMRMC despite the fact that we had an existing IACUC approval for this project. This IACUC approval was obtained from CSMC actually prior to funding of the grant. We were notified that we could not use any funds from this award until formal approval from USAMRMC had been obtained. This finally occurred in August 2005. This unfortunately delayed the initiation of our research efforts to some degree, but fortunately will not impact the execution of our studies. To compensate for the delay it is possible that we may request a no-cost extension of our studies (after year 4) to allow us to complete the phenotypic analysis of our mouse models.


Task 1: *In vitro* interactions of NF2 and HRS

Year 1: Co-immunoprecipitation using transfected cDNAs

We will transfect constructs used in the yeast two-hybrid screen after shuttling them into the pCDNA-HA vector. For co-ip experiments schwannomin will be precipitated using our anti-NF2 antibody (ab5991) using established procedures (Scoles et al 1998, 2000).

A good introduction to the subsequent studies is provided in the appended manuscript by Scoles et al. (2005), which describes initial results We realized that –in order to identify a dominant negative molecule- we needed to further define the cellular effects of different constructs shown in Fig. 1. Below are shown the cellular effects of several selected clones.

We realize that clone 4 is similar to a dominant-negative construct previously reported by Raiborg, et al. (rrr) and provides independent validation for our experiments.
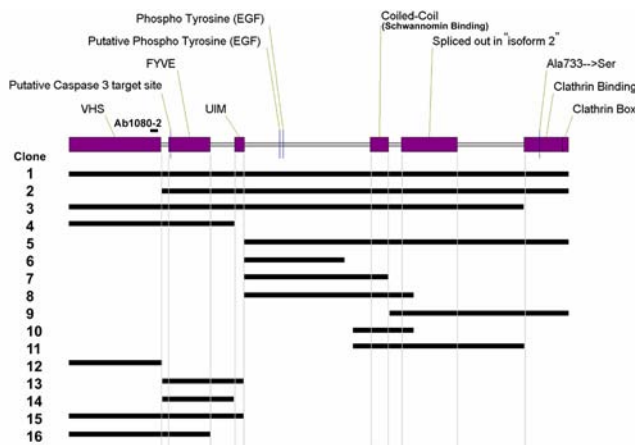

**Fig. 1: Schematic map of Hrs constructs**

We have generated and expressed all clones listed above. As examples, thwo clones are shown, which affect the distribution of EGFR, followed by an example of a clone that does not affect EGFR distribution.

DsRed-HRS(Clone4)   **EGFR-GFP**

Dox
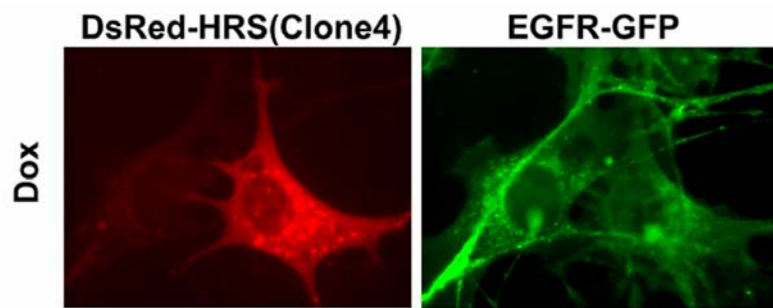
Fig. 2: Hrs4 disturbs epidermal Growth Factor Receptor (EGFR) trafficking when EGFR localization is compared between the transfected cell and the adjacent untransfected cell.

DsRed-HRS(Clone 14)   **EGFR-GFP**

Dox
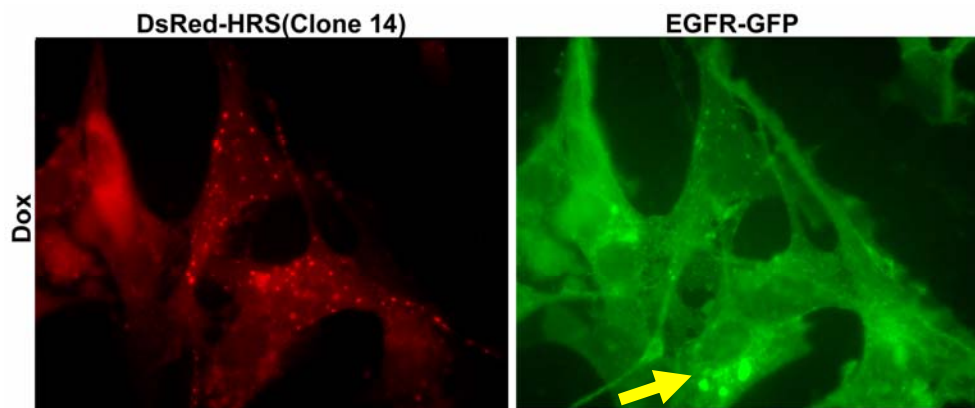
Fig. 3: Expression of clone 14 interferes with EGFR localization. Note the untransfected cell at bottom (yellow arrow), which show the typical accumulation of EGFR in enlarged endosome upon dox-stimulation (resulting in expression of wildtype Hrs).

DsRed-HRS(Clone 12)   EGFR-GFP

Dox

Dox

Dox
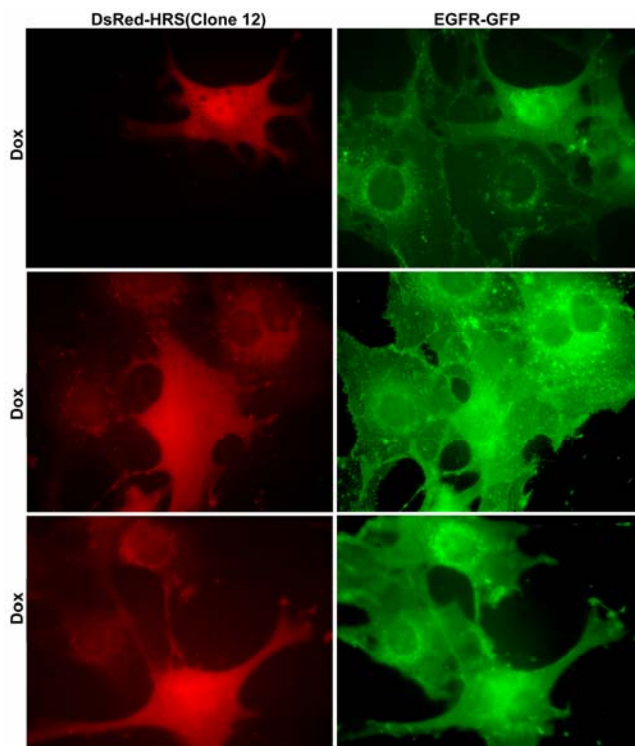
Figure 5: EGFR-GFP trafficking was not noticeably affected by DsRed-HRS clone 12 which localized diffusely and to no specific cellular region. Dox indicates cells treated with doxycycline, which results in induction of wildtype HRS.

Statement of work year 2

Year 2: Functional evaluation of HRS fragments

Since our initial submission of the grant proposal in 2002, the cellular biology field has been revolutionized by the ability to suppress gene expression using small inhibitory RNAs. We may slightly shift the focus of our 2nd year and attempt suppression of HRS using siRNA molecules. This would have the advantage that these effects may be more specific than the expression of truncated Hrs molecules.

Task 2: Effects of Hrs haploinsufficiency on *NF2$^{+/-}$* mice

Year 1: Hrs$^{+/-}$ intercross with Nf2$^{+/-}$

We will perform an intercross of Hrs$^{+/-}$ mice with Nf2$^{+/-}$ (mice Nf2$^{+/KO3}$). The F1 animals will be in a mixed background of 50%FVB/N and 50%C57BL/6J. As there is no evidence for segregation distortion for these alleles in previous publications, we expect to obtain the following genotypes: 25% Hrs$^{+/+}$;Nf2$^{+/+}$, 25% Hrs$^{+/+}$;Nf2$^{+/-}$, 25% Hrs$^{+/-}$;Nf2$^{+/+}$, 25% Hrs$^{+/-}$;Nf2$^{+/-}$.

Upon receipt of final approval from the USAMRMC on 8-22-06, we began setting up breeding colonies. As of April 30th, 2006, we have mated 37 pairs of *Hrs$^{+/-}$* mice with *Nf2$^{+/-}$*. This has resulted in 117 viable offspring. Animals are in a 50/50 C57B6/FVB background.

Offspring have a mean age of 4 months and are currently being followed for survival and development of tumors. One animal with an Nf2$^{+/-}$ genotype died, but had no visible tumors at autopsy. All other animals are aging without obvious signs of disease at this point as would be expected given their relatively young age.

We have found evidence of minor segregation distortion. Instead of the expected genotype ratios of 25% *Hrs$^{+/+}$;Nf2$^{+/+}$* , 25% *Hrs$^{+/+}$;Nf2$^{+/-}$*, 25% *Hrs$^{+/-}$;Nf2$^{+/+}$*, 25% *Hrs$^{+/-}$;Nf2$^{+/-}$*, we have found that wildtype animals are overrepresented with 37%, whereas the Nf2$^{+/-}$ genotype is underrepresented with only 17% of animals showing this genotype. There does not appear to be any segregation distortion regarding *Hrs* heterozygosity.

The finding of subtle segregation distortion is interesting as it has not been previously reported using these mouse lines despite extensive studies by other groups. It is conceivable that focus of prior groups was on tumor development and that this subtle segregation distortion was not worth reporting. On the other hand, it is possible this phenomenon is only visible with specific gene xgene or gene x environment interactions.

Task 3: *Hrs* knockout in Schwann cells

Year 2: Expression of a dominant negative Hrs *allele under the control of the P0 promoter*

As this task was planned for year 2 of the proposal, it has not yet been started. Due to the overall delay in funding initiation, we plan to begin this task in January 2007.

Key Research Accomplishments

- Dominant negative Hrs constructs have been generated.
- Intercrosses of $Hrs^{+/-}$ mice with $Nf2^{+/-}$ (mice $Nf2^{+/KO3}$) results in viable offspring.
- $Nf2^{+/-}$ mice appear to be born at slightly reduced rates.
- Tumor development does not appear to be greatly accelerated in double heterozygous animals at least to the extend that tumors are visible at 4 months.

Reportable Outcomes

Manuscripts published :

Scoles DR, Qin Y, Nguyen V, Gutmann DH, **Pulst SM**: HRS inhibits EGF receptor signaling in the RT4 rat schwannoma cell line.
Biochem Biophys Res Commun 335:385-392. (2005).

Conclusions

As the animal portion of this study has in effect only been conducted for about 6 months, it would be premature to report any firm conclusions. We did confirm, however, that Nf2/Hrs compound heterozygotes are viable. This provides further evidence that we will be able to carry out the proposed studies.

The observation of slight segregation distortion is interesting. At this point it is not clear whether this fact was indeed overlooked by prior investigators as they focused on tumor development or whether a special set of circumstances such as vivarium conditions or diet lead to increased death of $Nf2^{+/-}$ embryos or decreased fitness of $Nf2^{-}$ gametes. The segregation distortion will require a larger number of breeding pairs to obtain the required numbers for each genotype, but in the end will not affect our ability to carry out the proposed studies.

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APPENDICES

Scoles DR, Qin Y, Nguyen V, Gutmann DH, Pulst SM: (2005) HRS inhibits EGF receptor signaling in the RT4 rat schwannoma cell line. Biochem. Biophys. Res. Commun. 335: 385-392.

HRS inhibits EGF receptor signaling in the RT4 rat schwannoma cell line

Daniel R. Scoles [a,b,*], Yun Qin [a], Vu Nguyen [a], David H. Gutmann [c], Stefan-M. Pulst [a,b,d]

[a] *Rose Moss Neurogenetics Laboratory and Division of Neurology, Burns and Allen Research Institute, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA*
[b] *Department of Medicine, University of California at Los Angeles, Los Angeles, CA, USA*
[c] *Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110, USA*
[d] *Department of Neurobiology, University of California at Los Angeles, Los Angeles, CA, USA*

Abstract

Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is required for trafficking of cell surface receptors to the lysosome. Previously, we identified HRS as a protein that interacts with the neurofibromatosis 2 tumor suppressor schwannomin. In the present study, we established modified RT4 schwannoma cell lines that inducibly express HRS and constitutively express epidermal growth factor receptor (EGFR) fused to the green fluorescent protein. We demonstrated that HRS expression reduced EGFR abundance and EGF-mediated Stat3 activation. HRS expression also targeted EGFR to late endosomes. Schwannomin inhibited EGF-mediated Stat3 activation, consistent with HRS and schwannomin interacting in the same signaling pathway. Paradoxically, past studies have shown that HRS overexpression blocked EGFR trafficking to the late endosome and EGFR downregulation contrary to predictions of HRS function in HRS knockout studies. This study is the first to show that HRS can reduce the abundance of total and active EGFR and may reflect cell type-specific HRS function.
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Keywords: HRS; EGF receptor; EGFR trafficking; Schwannomin; Merlin; Neurofibromatosis 2; Vps27

Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is a regulator of endocytic trafficking of epidermal growth factor receptor (EGFR). We have previously identified HRS as a protein that interacts with the neurofibromatosis 2 (NF2) tumor suppressor schwannomin [1]. *NF2* gene inactivation is the cause for the inherited tumor syndrome NF2, and is also the causative genetic change in the majority of sporadic schwannomas and meningiomas. Several other proteins have been shown to interact with schwannomin including βII-spectrin, CD44, paxillin, NHERF, Rho GDI, and syntenin [2], but no single pathway has yet been associated with NF2 pathogenesis.

Aberrant EGFR expression is common in Schwann cell tumors and in tumors arising from *NF2* mutation. These include malignant peripheral nerve sheath tumors (MPNSTs) and neurofibromas, meningiomas, and schwannomas [3–5]. In addition, EGFR is overexpressed in 45% of mesotheliomas and schwannomin loss by *NF2* gene mutation increases patient risk of mesothelioma [6]. Understanding EGFR pathways in Schwann cell and other NF2-related tumors is important to defining therapeutic targets.

Previous studies have shown that HRS is required for EGFR internalization while, paradoxically, overexpressed HRS had an inhibitory effect on EGFR trafficking and downregulation. Yeast and *Drosophila* HRS knockout studies have established that HRS is required for trafficking of EGFR from the early-to-late endosome

[7–9]. EM analysis also showed that HRS-deficient *Drosophila* cells had enlarged multivesicular bodies (MVBs) that failed to properly invaginate [7], consistent with findings in yeast showing that HRS is required for receptors to traffic into the lumen of the MVB [9]. These findings were supported by HRS antisense studies demonstrating that HRS knockdown in HeLa cells increased EGFR signaling [10,11]. However, other studies have shown that HRS overexpression prevented EGFR downregulation, causing EGF or EGFR accumulation mainly in the early endosome with little localization of EGFR to late endosome/lysosome [12–15].

In our previous work, we demonstrated that common pathways of growth factor signaling could be inhibited by both HRS and schwannomin in schwannoma cell lines relevant to the NF2 phenotype. We showed that both schwannomin and HRS were able to inhibit IGF-I-mediated proliferation and Stat3 phosphorylation in human STS26T schwannoma and RT4 rat schwannoma cell lines [16]. We also showed a requirement for HRS expression for schwannomin to inhibit growth of mouse embryo fibroblasts [17].

To test the hypothesis that HRS inhibits EGFR in cells relevant to NF2, we developed schwannoma cell lines that stably express EGFR fused to the green fluorescent protein (GFP) that are inducible for HRS or schwannomin expression. We found that induced HRS expression in RT4 schwannoma cells trafficked EGFR to the late endosome and reduced abundances of total and active EGFR after EGF treatment. While HRS trafficked EGFR to the late endosome, schwannomin was unable to traffic EGFR but had a similar effect as HRS on inhibition of EGF-activated Stat3 phosphorylation.

Materials and methods

Cell lines. HREG cells were made by transfecting pEGFR-GFP [18] into Tet-on RT4-HRS10 [19] cells along with pTK-HYG (Clontech) and selecting with 400 μg/ml hygromycin (Gibco). Lines inducible for schwannomin isoform 1 expression were generated following the same approach as for HREG cells but starting with Tet-on RT4-NF2.17 cells [19].

Antibodies. Rabbit polyclonal anti-HRS antibody ab1080-2 was described previously [1]. Other antibodies included Xpress mAb (Invitrogen), GFP mAb (Chemicon), β-Cop mAb (Sigma), EEA1 mAb (Transduction Laboratories), goat anti-LAMP2 (Santa Cruz), active EGFR mAb (Transduction Laboratories), EGFR mAb (Santa Cruz), anti-phospho-Stat3 (Cell Signaling Technology), polyclonal Stat3 antibody (Cell Signaling Technology), and anti-actin AC-40 (Sigma). Secondary antibodies included donkey anti-rabbit or mouse conjugated to TRITC or FITC and goat anti-rabbit, mouse or chicken conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories). The secondary antibodies used for triple-immunofluorescent detections were Alexa Fluor 594 donkey anti-mouse or rabbit, and Alexa Fluor 647 donkey anti-goat (Molecular Probes).

Internalization assay. Equal cultures of HREG cells were plated in 48 1.9 cm^2 wells in DMEM + 10% FBS and grown overnight. The next day an equal volume of media containing 2 μg/ml dox was added for a final dox concentration of 1 μg/ml and the cells were grown overnight.

For "no dox" controls, an equal volume of dox diluent (water) was added. The next evening the media were changed to DMEM + 0.5% FBS and the cells were incubated overnight with the same conditions dox. The next morning the media in each of the cultures were rinsed with 0.2 ml 37 °C binding buffer (DMEM + 0.1% BSA). Cultures were treated in quadruplicate with 0.06 μCi of ^{125}I-labeled EGF (Amersham) in 130 μl 37 °C binding buffer for the indicated times, then placed on ice, and washed three times with cold DMEM, 1 ml per wash. Surface-bound ^{125}I-labeled EGF was eluted from the cells by incubating cultures in elution buffer (0.2 M acetic acid, 0.5 M NaCl, pH 2.8) for 5 min. Internalized ^{125}I-labeled EGF was collected by solublizing cells in 1 M NaOH overnight.

Pulse-chase assays. Equal cultures of HREG cells were plated in 10 cm dishes and grown overnight. The next day an equal volume of media containing 2 μg/ml dox was added for a final dox concentration of 1 μg/ml and the cells were grown overnight. For "no dox" controls, an equal volume of dox diluent (water) was added. The next evening the media were changed to DMEM lacking FBS and the cells were serum-starved 10 h with the same conditions dox in a total volume of 4 ml. The untreated plates were harvested and all others were treated with EGF by adding 4 ml of 2× EGF for a final concentration of 100 ng/ml. Controls were treated in the same manner with EGF carrier consisting of 0.1 mM acetic acid. After 10 min of EGF treatment, the plates with zero chase time were harvested and the others were washed three times in 37 °C serum-free DMEM and incubated at 37 °C in serum-free DMEM containing 100 mg/ml cycloheximide for the indicated times. Cells were harvested in SDS–PAGE buffer and analyzed by immunoblotting.

Immunofluorescent labeling and microscopy. Cells were plated on poly-L-lysine-coated glass coverslips, treated with dox or EGF as indicated, and were immunofluorescently labeled as previously described [20,21]. Standard immunofluorescent detection was used to capture the images in Figs. 1A, D, and E, and 2A and D using a Zeiss Axiovert 100 M microscope equipped with a Spot2 camera, objective Plan-Apochromat 63×/1.4 (Zeiss). The images in Figs. 2B and C, and 5C were captured by fluorescent confocal microscopy performed using a Leica TCS SP confocal microscope, objective Plan-APO 100×/1.40 (Leica). GFP was excited with a ArKr laser at 488 nm with emission set to a range of 503–508 nm. Alexa Fluor 594 was excited with a ArKr laser at 594 nm with emission set to a range of 580–649 nm. Alexa Fluor 647 was excited with a HeNe laser at 633 nm with emission set to a range of 649–719 nm.

Results

Characterization of HREG cells

HRS was overexpressed in HREG cells by doxycycline treatment. Induced Xpress-epitope-tagged HRS was detected by immunofluorescent labeling with an anti-Xpress antibody (Fig. 1A). No background labeling was observed with anti-Xpress in controls not treated with dox (Fig. 1A). Dox-induced cells labeled with anti-HRS antibody 1080-2 showed HRS induction, and in cells not treated with dox the background of endogenous HRS was observed (Fig. 1A). Immunoblotting revealed a strong specific HRS band in induced extracts compared to endogenous HRS in extracts that were not induced (Fig. 1B).

The pEGFR-GFP plasmid encodes an active GFP-tagged EGFR that internalizes upon EGF binding [18]. Immunofluorescent labeling of the exogenous
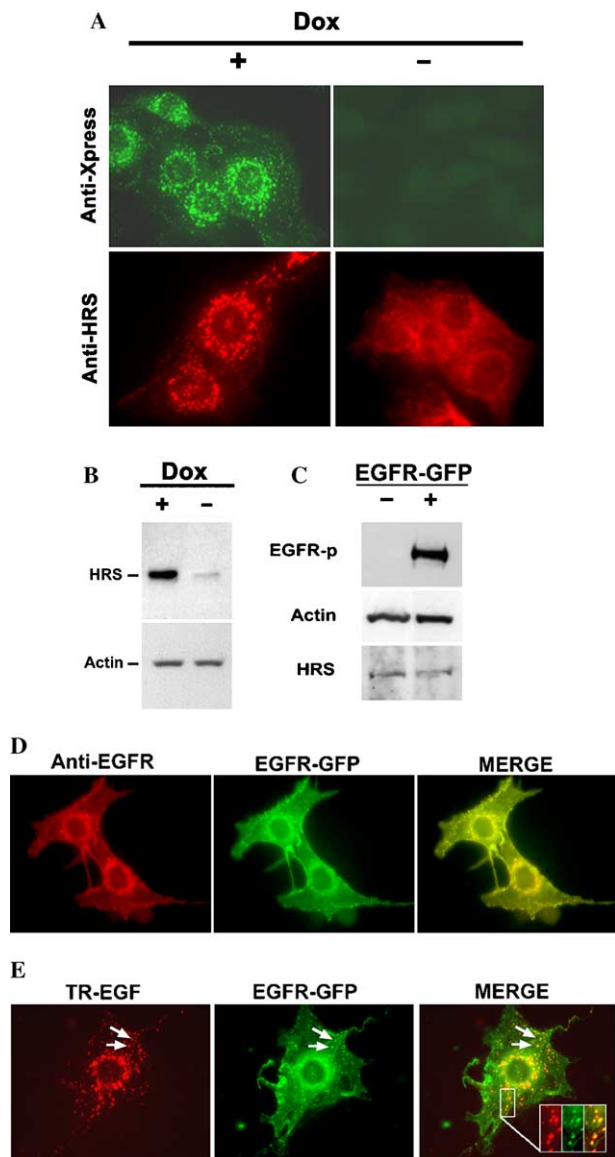
Fig. 1. Characterization of HRS and EGFR-GFP in HREG cells. (A) Induction of HRS by addition of 2 μg/ml dox (+) in HRS-inducible Tet-on RT4 cells before EGFR-GFP transfection compared to uninduced cells (−), followed by immunofluorescent labeling with anti-Xpress or anti-HRS antibodies. Note that HRS was highly expressed compared to endogenous HRS. (B) Cell extracts from induced (+) or uninduced (−) cells detected by immunoblotting with anti-HRS and anti-actin antibodies. (C) Phosphorylation of EGFR in HRS-inducible Tet-on RT4 cells before (−) and after (+) stable transfection of EGFR-GFP by treatment of serum-starved cells with 100 ng/ml EGF. (D) HREG cells were immunofluorescently labeled with anti-EGFR mAb antibody and secondarily labeled with rhodamine-conjugated donkey anti-mouse antibody. Immunofluorescent microscopy demonstrated complete co-localization of rhodamine immunofluorescence with EGFR-GFP. (E) EGFR-GFP in HREG cells co-localized with Texas Red (TR)-conjugated EGF (arrows). Cells were treated with 100 μg/ml TR-EGF for 15 min and were not treated with doxycycline.

EGFR-GFP with anti-EGFR antibody demonstrated complete co-localization showing that the pEGFR-GFP construct properly expressed EGFR (Fig. 1D).

Texas Red-conjugated EGF stimulated EGFR-GFP internalization and EGFR-GFP strongly co-localized with Texas Red EGF demonstrating ligand binding by EGFR-GFP (Fig. 1E). EGFR-GFP expressed in RT4 cells treated with EGF was detected by immunoblotting using anti-EGFR-P antibody demonstrating that EGFR-GFP was phosphorylated by EGF in our model system (Fig. 1C).

HRS induction trafficked EGFR-GFP

EGFR-GFP trafficked to late endosomes when HRS was overexpressed. When we treated HREG cells overnight in dox media, EGFR-GFP accumulated in perinuclear vesicles (Fig. 2A). Since HRS had been reported to localize mainly in early endosomes [14], the appearance of perinuclear structures upon HRS induction containing EGFR-GFP but lacking HRS (Fig. 2B) is consistent with EGFR-GFP localization to the late endosome. Perinuclear structures containing EGFR-GFP labeled positively with the late endosome/lysosome marker LAMP2 and partially co-localized with the early endosome marker EEA1 (Fig. 2C). High-magnification images showed that the greatest accumulations of EGFR-GFP co-localized with the strongest labeling for LAMP2 in structures lacking EEA1 labeling (Fig. 2C). Analysis of β-Cop labeling showed that EGFR-GFP did not traffic to the Golgi compartment (Fig. 2D).

HRS effects on EGFR and Stat3 activation

Since overexpression of HRS resulted in EGFR-GFP trafficking to a structure labeling positive for LAMP2, we predicted that HRS expression would inhibit EGF signaling. To test this, we examined the effect of HRS on the abundances of active EGFR and Stat3 taking full advantage of our inducible HREG cells to monitor a dose response. We treated HREG cells with EGF or carrier after induction of HRS with varying doses of dox. Immunoblotting with lanes loaded such that all had even amounts of total EGFR-GFP showed that cells possessed less active EGFR relative to total EGFR-GFP when HRS was overexpressed (Fig. 3A). Initially not considered a target of EGFR, Stat3 is now well established as such, and active Stat3 dimers are common in several tumor types with EGFR defects [22,23]. We demonstrated that dosewise increases in HRS expression inhibited EGF activation of Stat3 (Figs. 3B and C).

To understand whether the HRS effect on EGFR or Stat3 activation by EGF might be related to the abundance of receptors on the surface, we measured ligand internalization by HREG cells with different conditions of HRS induction. We incubated cells with ^{125}I-labeled EGF for increasing times and then measured surface-bound and internalized ^{125}I-labeled EGF. HREG cells were less able to uptake ^{125}I-labeled EGF when HRS
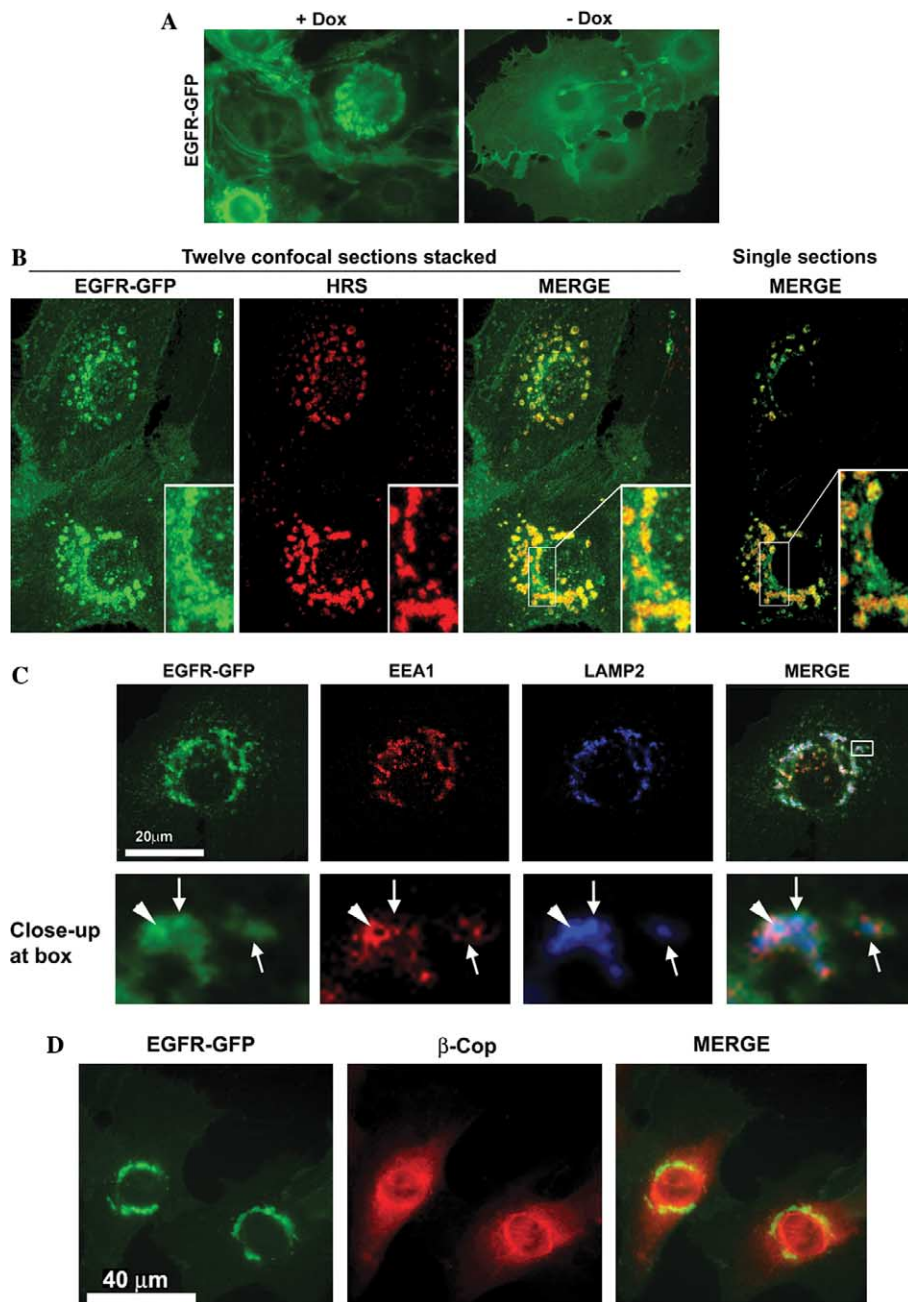
Fig. 2. The overexpression of HRS in HREG cells was associated with EGFR-GFP trafficking to the LAMP2-positive compartment adjacent to the Golgi compartment. (A) HRS induction (2 µg/ml dox) caused EGFR-GFP accumulation in a perinuclear compartment not seen in uninduced cells. (B) Not all perinuclear structures that formed when HRS was induced by overnight treatment with dox were positive for HRS labeling. Serum-starved cells were induced with EGF for 10 min. Inset, 2.5× magnification. (C) In cells treated with dox overnight to induce HRS and labeled with anti-EEA1 and anti-LAMP2, high magnification (box) revealed regions where EGFR-GFP co-localized with LAMP2 but not EEA1 (arrows). Some EGFR-GFP/LAMP2-positive structures were surrounded by EEA1 labeling (arrowhead). (D) Trafficked EGFR-GFP did not localize with the pre-Golgi marker antibody β-Cop. Standard immunofluorescent microscopy (A,D), confocal microscopy (B,C).

was induced (Fig. 3D), consistent with the presence of fewer EGFR receptors at the cell surface when HRS was overexpressed.

HRS effects on EGFR abundance

To determine the effect of HRS on EGFR abundance, we overexpressed HRS in HREG cells and extracted pro-

teins for analysis of EGFR by immunoblotting. In six independent replications, we observed that overnight HRS induction had no effect on EGFR abundance in HREG cells cultured in media containing serum (Figs. 4A and B). However, when we serum-starved HREG cells and then treated them with a short pulse of EGF, we observed that when HRS was present the abundance of EGFR-GFP was rapidly reduced (Figs. 4B and C). This
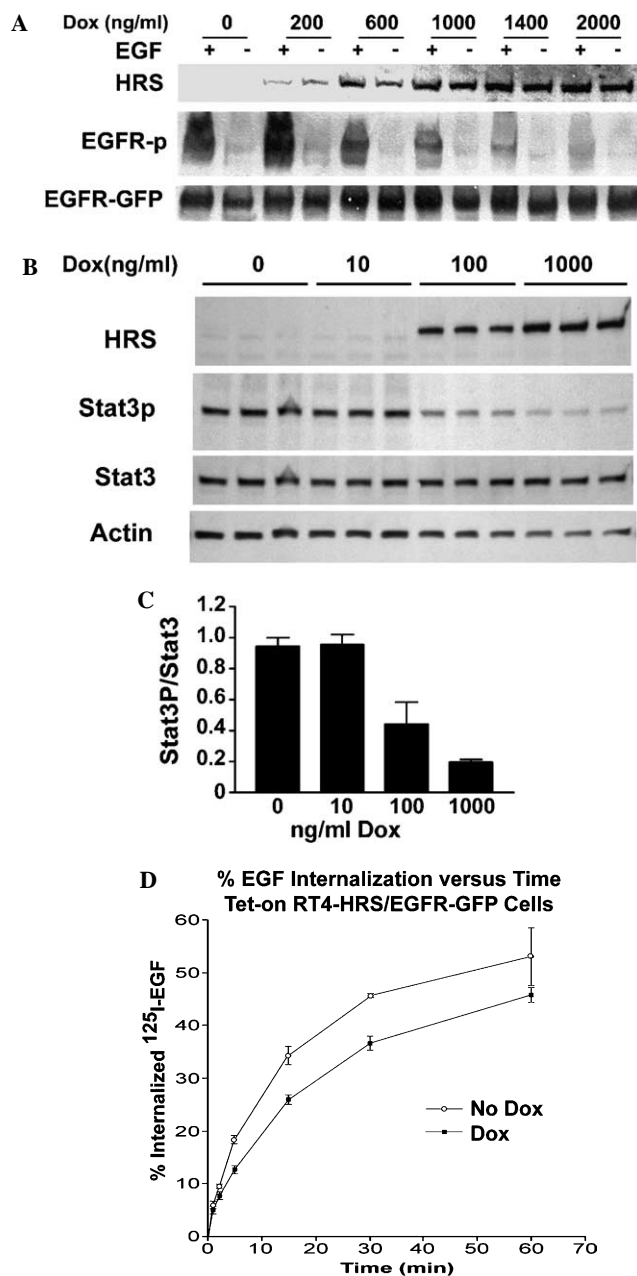
Fig. 3. HRS inhibits EGFR signaling in HREG cells. (A) HRS induction reduced EGF-mediated EGFR phosphorylation. Cells were treated with 100 ng/ml EGF for 15 min. Activated EGFR (EGFR-P) was detected on immunoblots with anti-phospho EGFR antibody. Note that loading was normalized to EGFR-GFP using anti-GFP and this immunoblot provides no information on HRS effect on the total abundance of EGFR-GFP. (B) HRS induction inhibited EGF-mediated Stat3 phosphorylation. Triplicate cultures of HREG cells induced with doxycycline were serum-starved, treated with 100 ng/ml EGF for 15 min, and then the cell extracts were examined by immunoblotting for total and active Stat3. (C) Densitometric analysis of the immunoblot in (B) showing relative changes in active Stat3. (D) HRS decreased the internalization of ^{125}I-labeled EGF. Cells were treated with ^{125}I-labeled EGF for the indicated times at 37 °C, chilled, then unbound ^{125}I-labeled EGF was removed, and the surface-bound and internalized EGF was determined by an acid wash protocol. Decreased internalization was observed when HRS was overexpressed by the addition of dox (two-factor ANOVA $P < 0.001$). Values are means and standard deviations from four replicates.
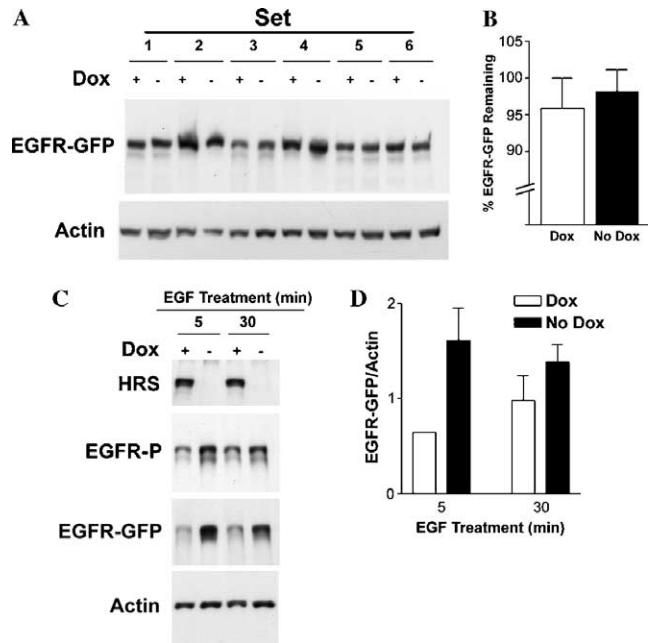

Fig. 4. EGFR abundance is reduced when HRS is expressed only after a pulse of EGF. (A) Six different pairs of HREG cell cultures were treated with (+) or without (−) 1 μg/ml dox overnight and EGFR-GFP abundances were assessed by immunoblotting. (B) Densitometric analysis of the immunoblot in (A) where the highest ratio of EGFR-GFP/actin for each pair was assigned 100%. One-way ANOVA demonstrated that HRS had no significant effect on EGFR abundance ($P > 0.05$). (C) HREG cells were treated with (+) or without (−) 1 μg/ml dox overnight, serum-starved 10 h, and then treated with 100 ng/ml EGF for the indicated times. Levels of total and active EGFR were determined by immunoblotting. (D) Densitometric analysis of the immunoblot in (C) demonstrated that EGFR-GFP abundance was reduced when HRS was overexpressed. Two-factor ANOVA demonstrated a significant difference between the conditions of + or − dox ($P < 0.05$), but not between the chase times ($P = 0.74$).

rapid change in EGFR abundance when HRS was present suggested that the reductions in active EGFR and Stat3 that we observed in Fig. 3 were related to HRS-mediated reductions in total EGFR abundance in addition to HRS-mediated reduction in the number of EGFR surface receptors. Furthermore, these results demonstrate that HRS overexpression reduces EGFR-GFP abundance in HREG cells only after a pulse of EGF.

Pulse/chase studies

HRS overexpression did not reduce EGFR abundance after a pulse of EGF followed by a chase. We pulsed HREG cells treated with or without dox to induce HRS with EGF and then chased with cycloheximide media. HRS expression was associated with reduced abundance of total EGFR after stimulation by EGF, but not after 90 or 180 min chasing (Figs. 5A and B). When we observed cells treated in the same manner by immunofluorescent microscopy, the fate of EGFR-GFP depending upon HRS and EGF became
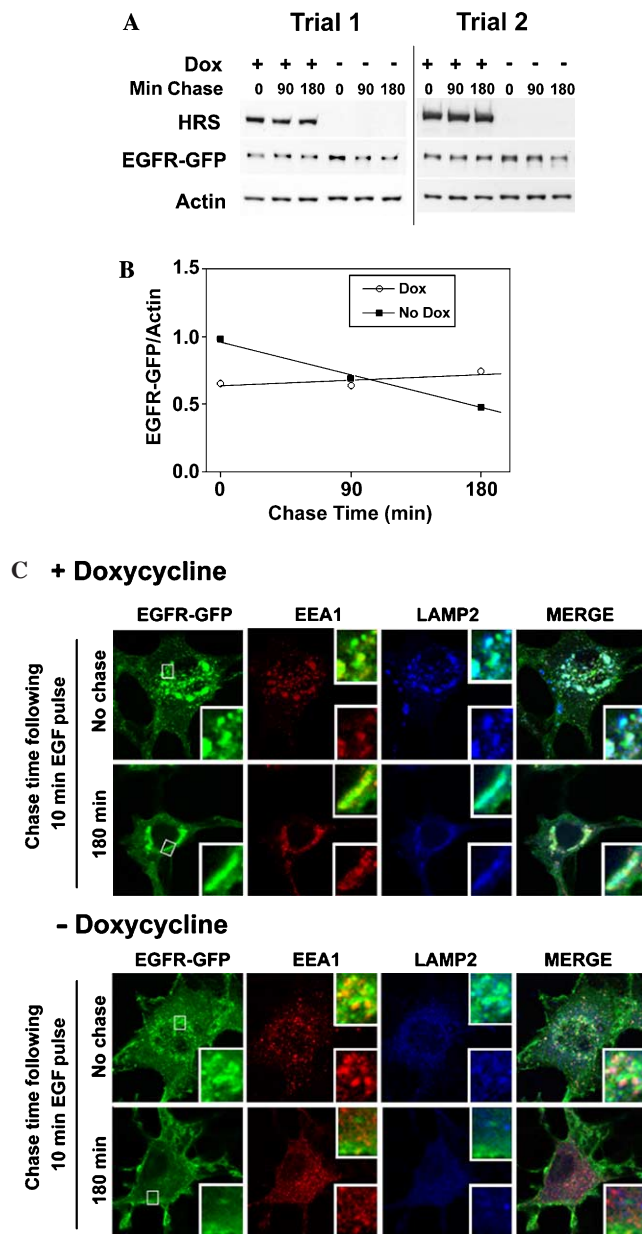
A

B

C + Doxycycline

– Doxycycline

Fig. 5. EGFR abundance is not reduced by HRS overexpression after chasing. (A) Cells were treated with (+) or without (−) 1 µg/ml dox overnight, serum-starved, pulsed 10 min with 100 ng/ml EGF, and chased in media containing 100 mg/ml cycloheximide for the indicated times. Levels of total and active EGFR were determined by immunoblot analysis relative to actin. (B) Plots of EGFR-GFP/actin vs chase time determined densitometrically from the immunoblots in (A). Averages of the two trials are plotted. Regression analysis showed that the slopes of the lines are significantly different ($P < 0.05$). (C) Cells treated in the same manner as those in (A) evaluated by confocal immunofluorescent microscopy for EGFR-GFP, EEA1, and LAMP2 localization. Images are 50 µm across. Insets magnified four times. For EEA1 and LAMP2, merged images with EGFR-GFP are also shown in insets at the top-right of each frame.

clear. There were two types of vesicles in cells expressing HRS pulsed with EGF, including those that had clustered around the nucleus in response to HRS overex-

pression overnight under serum-starved conditions, and small vesicles that had newly internalized during the EGF treatment (Fig. 5C). After the chase the newly internalized vesicles were eliminated and only a cluster of EGFR-containing early or late endosomes near the nucleus remained (Fig. 5C). However, when HRS was not overexpressed, vesicles that had internalized during the EGF treatment were eliminated after chasing and the only remaining EGFR-GFP was that at the membrane that did not internalize during the pulse (Fig. 5C).

Schwannomin inhibits Stat3 phosphorylation by EGF

Our interest in studying HRS ability to inhibit EGFR signaling in a schwannoma cell line relates to our previous demonstration that HRS interacts with the NF2 tumor suppressor protein schwannomin which is important for the pathogenesis of tumors of Schwann cell origin [1]. We also assessed the ability for schwannomin to regulate EGFR trafficking in a cell line model inducible for schwannomin expression. Schwannomin-inducible Tet-on RT4 NF2/EGFR-GFP cells treated with EGF and different doses of doxycycline demonstrated that schwannomin expression inhibited EGF-mediated Stat3 phosphorylation (Fig. 6). However, induced schwannomin expression had no effect on triggering the internalization of EGFR-GFP in two schwannomin-inducible Tet-on RT4 NF2/EGFR-GFP cell lines (data not shown).
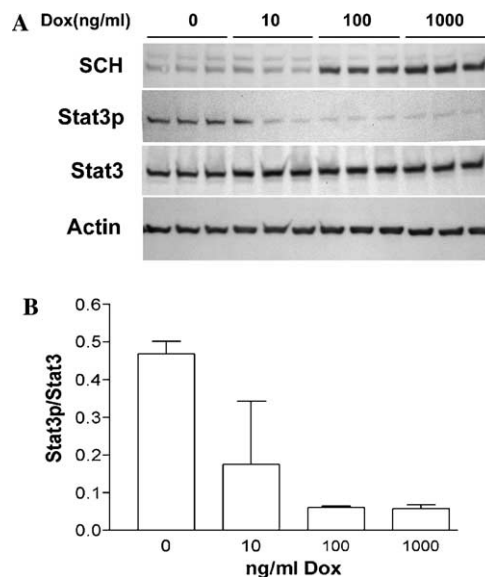
A

B

Fig. 6. Schwannomin induction inhibited EGF-mediated Stat3 phosphorylation in schwannomin-inducible Tet-on RT4/EGFR-GFP cells. (A) Triplicate cultures induced with doxycycline were serum-starved, treated with 100 ng/ml EGF, and then the cell extracts were examined by immunoblotting for total and active Stat3. Schwannomin was detected using anti-NF2 antibody A-19 (Santa Cruz). (B) Active Stat3 changes corresponding to (A).

Discussion

Previous reports have demonstrated that HRS is required for EGFR internalization [7–11] while, paradoxically, overexpressed HRS had an inhibitory effect on EGFR trafficking and downregulation [12–15,24]. Our data confirmed that HRS expression inhibited EGF activation of EGFR and Stat3, but we also observed that HRS expression reduced the abundance of active and total EGFR after EGF activation.

HRS inhibits EGFR and Stat3 activation by EGF

HRS overexpression was effective for downregulating EGFR in HREG cells. To assess the ability for HRS to inhibit EGFR signaling, we titrated doxycycline to incrementally increase HRS abundance and assessed phosphorylation of EGFR and Stat3 in response to EGF treatment. HRS proved to be a potent inhibitor of EGF-mediated EGFR and Stat3 activation. Increases in HRS abundance were associated with decreases in the levels of phosphorylated EGFR and Stat3 (Fig. 3). The reduced ability for cells to uptake ^{125}I-labeled EGF when HRS was overexpressed suggested that HRS reduced the number of receptors at the cell surface. This conclusion is consistent with our observation that HRS trafficked EGFR-GFP to perinuclear structures in HREG cells even when cells were serum-starved for 24 h before the addition of doxycycline (data not shown), and a recent report that HRS overexpression resulted in the internalization of unstimulated EGFR [24]. Reductions in the number of EGF receptors at the cell's surface could explain the reduced EGF-mediated EGFR and Stat3 phosphorylation that we observed when HRS was overexpressed. This led us to investigate the action of HRS on total EGFR abundance.

HRS had no effect on the abundance of EGFR-GFP in HREG cells that were not subjected to a pulse of EGF (Figs. 4A and B). This was not entirely surprising since it was previously shown that HRS is required for the specific degradation of the active form of EGFR by investigation of Hrs knockout flies [7], but also because no one else had previously demonstrated that HRS overexpression reduced EGFR abundance. However, when we serum-starved HREG cells and then treated them with EGF for as little as 5 min, we observed a significant reduction in the abundance of EGFR-GFP (Figs. 4C and D).

We used the pulse/chase strategy to further assess the HRS effect on EGFR abundance. We observed that a 10-min pulse of EGF resulted in reduced EGFR-GFP abundance in cells overexpressing HRS, but EGFR-GFP abundance was not further reduced after chasing. These experiments were consistent with our microscopic observations. Immediately after stimulation we observed GFP-positive vesicles trafficking from the plasma membrane. After chasing, internalized GFP fluorescence was eliminated in cells not overexpressing HRS. But in cells overexpressing HRS the small newly internalized vesicles were eliminated and the only GFP fluorescence remaining was that in large vesicles that had internalized during the overnight serum starvation. We also noted the greatest degree of EGFR-GFP co-localization with LAMP2 in cells induced to overexpress HRS immediately after a pulse of EGF (Fig. 5C). Because we demonstrated that HRS reduced EGFR abundance only in the presence of EGF (Fig. 4), we concluded that HRS facilitated the degradation of activated EGFR after a pulse of EGF, and that the rapidly internalizing vesicles that appeared after a pulse of EGF were facilitated by HRS to deliver EGFR to the late endosome.

Our findings that HRS prevents EGFR degradation after prolonged chasing are consistent with previous investigations. However, this study is the first to show that HRS overexpression can reduce EGFR abundance after a pulse of EGF. The effect by HRS to inhibit EGFR degradation during the chase we speculate may be because EGFR that had trafficked during the overnight HRS induction was inaccessible to stimulation by EGF and was not further trafficked since it remained inactive. Other studies have provided insight into the mechanism for how HRS prevents EGFR degradation. EGFR trafficking may be halted at the early endosome as long as HRS is present on vesicles and interacted with PtdIns(3)P [14]. Furthermore, EGFR trafficking may be halted at the early endosome until HRS interacts with a release factor dissociating it from the endosome [25]. Such a release factor might become depleted in systems where HRS is highly overexpressed, resulting in EGFR-GFP accumulations in the endosomal system.

Schwannomin inhibits Stat3 activation by EGF

Previously, we showed that schwannomin and HRS function similarly to inhibit RT4 cell proliferation [16,19]. We have now shown that schwannomin inhibited EGF-activated Stat3, much like HRS (Fig. 6). Since HRS and schwannomin interact and co-localize at early endosomes [1], we hypothesized that schwannomin would inhibit EGFR signaling by altering EGFR trafficking. Schwannomin, however, did not. As the ability of schwannomin to inhibit cellular proliferation is dependent on the presence of HRS [2,17], schwannomin may require HRS-mediated vesicle internalization to inhibit Stat3 phosphorylation downstream of EGFR. Alternatively, schwannomin and HRS may interact in a novel pathway that remains to be elucidated.

This study presents for the first time the ability for overexpressed wildtype HRS to inhibit EGFR signaling. We conclude that the reductions in EGFR mediated by HRS overexpression represent the normal HRS in vivo function and is either specific to Schwann or schwannoma cells or has never before been observed in other cell

types because EGFR changes have not been assessed immediately after a pulse of EGF. The HREG cell line may prove useful for the analysis of schwannomin or other HRS effectors on regulating HRS in ways that are not possible with HRS knockout systems.

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